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TECHNICAL MANUSCRIPT 447

FRACTIONATION OF EASTERN EQUINE ENCEPHALITIS VIRUS BY DENSITY GRADIENT CENTRIFUGATION IN CsCl

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DEPARTMENT OF THE ARMY
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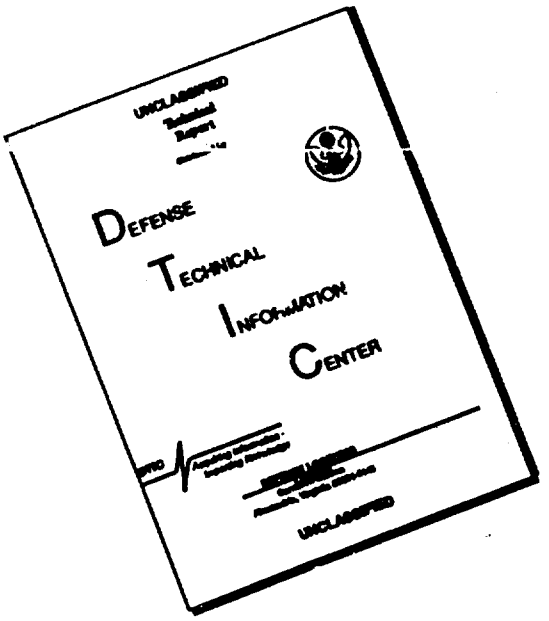

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ABSTRACT

Experiments in which partially purified eastern equine encephalitis (EEE) virus was centrifuged to equilibrium in CsCl revealed three bands. These consisted of a hemagglutinating particle, ρ = 1.18; a major infectious band, ρ = 1.20; and a minor infectious band, ρ = 1.23.

Analysis of radioactive profiles of CsCl-fractionated EEE, labeled with either $P^{32}O_4$ or uridine-H^3, indicated that most of the hemagglutinin was stripped from the complete virion. The viral origin of the hemagglutinin was verified by inhibition with specific antiserum.

Attempts to differentiate EEE ρ = 1.23 from the complete virion (ρ = 1.20) showed that the heavier particle was neither a viral contaminant, a density mutant, nor an immature form of the virus. Results from a CsCl rebanding experiment support the fact that EEE ρ = 1.23 was produced by a CsCl-induced alteration or degradation of the complete virion.

I. INTRODUCTION

Two representative arboviruses, Sindbis[1,2] and dengue 2,[3] have been characterized with respect to their buoyant densities in CsCl. With both viruses, marked heterogeneity in density was apparent, and, in some cases, antigenic components could be resolved from the major band of infectious virus. The arbovirus hemagglutinating particle is considered to be identical with the virion and is thought to be located in the envelope of the virus.[4]

In this report, we have determined that the buoyant density (ρ) of the group A arbovirus eastern equine encephalitis (EEE) is 1.20 g/cm^3. In addition, two other bands of viral material were observed: a light hemagglutinin (HA) ρ = 1.18 and infectious virus of a higher density (ρ = 1.22 to 1.23). These two bands appear to be the salt-induced breakdown products of the EEE virion.

II. MATERIALS AND METHODS

The Louisiana strain of EEE, the origin and properties of which were reported by Brown,[5] was used in all experiments. The virus was propagated on chick embryo monolayers and assayed by a plaque technique described by Zebovitz and Brown.[6]

Prior to density gradient centrifugation, EEE was partially purified from cell culture fluid that had been decanted from infected cells and adsorbed onto $AlPO_4$ gel.[7] The virus was eluted from the gel in 0.3 M phosphate buffer, pH 8.0, and concentrated by centrifugation at 65,000 x g for 60 minutes. The resulting pellet was resuspended by allowing it to stand overnight in borate-saline buffer, pH 9.0,[8] containing 0.1% bovine serum albumin. Typically, only 15 to 20% of the total plaque-forming units (pfu) were recovered; however, an overall purification of as high as 500-fold was obtained (2.0×10^{10} pfu/mg protein).

CsCl was added to the partially purified EEE virus to ρ = 1.21. The virus was unstable in the salt if the bovine serum albumin was omitted from the buffer. Centrifugation was carried out at 36,000 rpm for 24 hours using an SW-39 rotor and a Spinco Model L centrifuge. Following centrifugation, five-drop fractions (0.05 ml) were collected from the bottom of the tube, diluted 1:10 with beef heart infusion broth, and assayed. Generally, every tenth fraction was held undiluted for the determination of its refractive index. Density was calculated from the empirical formula $\rho^{25°} = 10.8601(n_D^{25°}) - 13.4974$.

Labeled EEE virus was prepared by including 10 μc $P^{32}O_4$/ml or 5 μc uridine-H^3/ml in the virus growth medium. Actinomycin D (2 μg/ml) was also included when uridine-H^3 was employed. All radioactivity measurements represent cold trichloroacetic acid - insoluble virus material trapped on membrane filters. The dried filters were counted in toluene-based BBOT [2,5-bis-2(5-tert-butylbenzoxayoyl)-thiophene] using a Packard liquid-scintillation spectrometer. Titrations for HA activity were carried out according to the method of Clarke and Casals[8] in microtiter trays.

III. RESULTS

Visual examination of banded EEE virus revealed three bands: the uppermost band was diffuse, the middle band was well defined and contained the majority of the banded material, and the lower band was sometimes composed of two distinguishable, although poorly resolved, bands. The latter were considered as one band because the two minor bands did not always appear and were, in all cases, inseparable during fractionation. The majority of the pfu (82%) were in the mid-band, $\rho = 1.20$ (Fig. 1). Significant titratable virus was also detected in a saddle-shaped peak in the density range $\rho = 1.22$ to 1.23. All plaques produced from these various fractions were of the same size and morphology as those of parental EEE virus. Coincident with the peaks of infectious virus, significant HA titers were found. These values represented only 10% of the HA titers located in fractions 48 to 50 ($\rho = 1.18$). While the maximum HA titer was in the least dense band, the latter contained only 3% of the pfu titer.

In the next experiment, EEE virus labeled with uridine-H^3 was centrifuged through a CsCl gradient. Under these conditions, only particles containing RNA would be detected in the radioactivity profile. The results summarized in Figure 2 suggest that the HA activity found at $\rho = 1.18$ may be attributed to either fragments of the viral envelope or a particle split from the surface of the envelope because no H^3 was detected. Proof that the HA material banding at $\rho = 1.18$ was virus-specific was afforded by its inhibition by antiserum in HA inhibition tests.

The CsCl banding pattern described above was observed in every experiment, and, although the separation of the HA material from the EEE virion ($\rho = 1.20$) was anticipated, the infectious virus found at $\rho = 1.22$ to 1.23 was unexpected. It was important to determine whether this denser species was a virus contaminant, a density mutant, an immature or subviral form of EEE, or a breakdown product of the complete EEE virion.

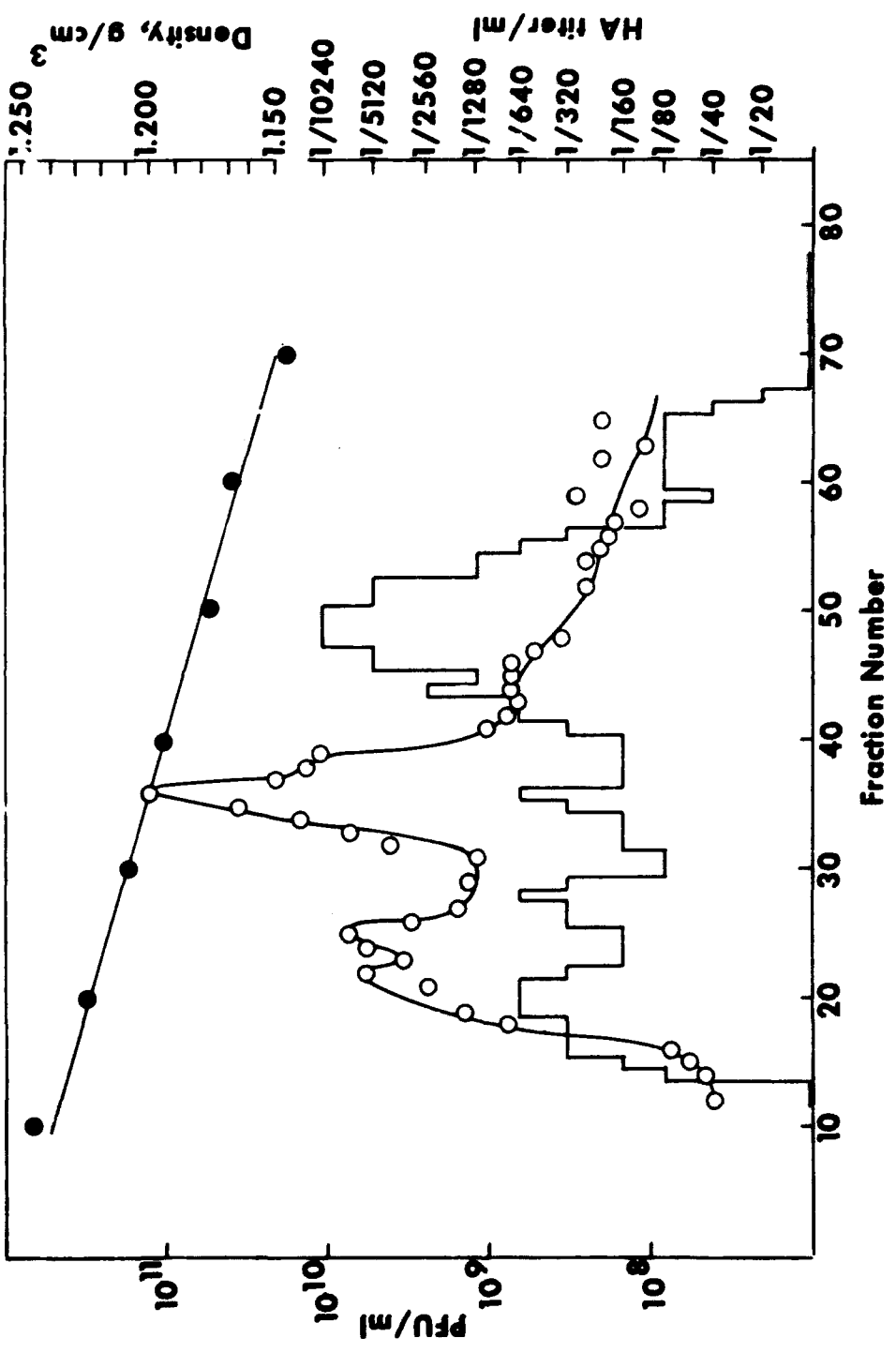

Figure 1. CsCl Density Gradient Centrifugation of Partially Purified EEE Virus. Virus infectivity (o), CsCl density (•), hemagglutinin titer (bar diagram).

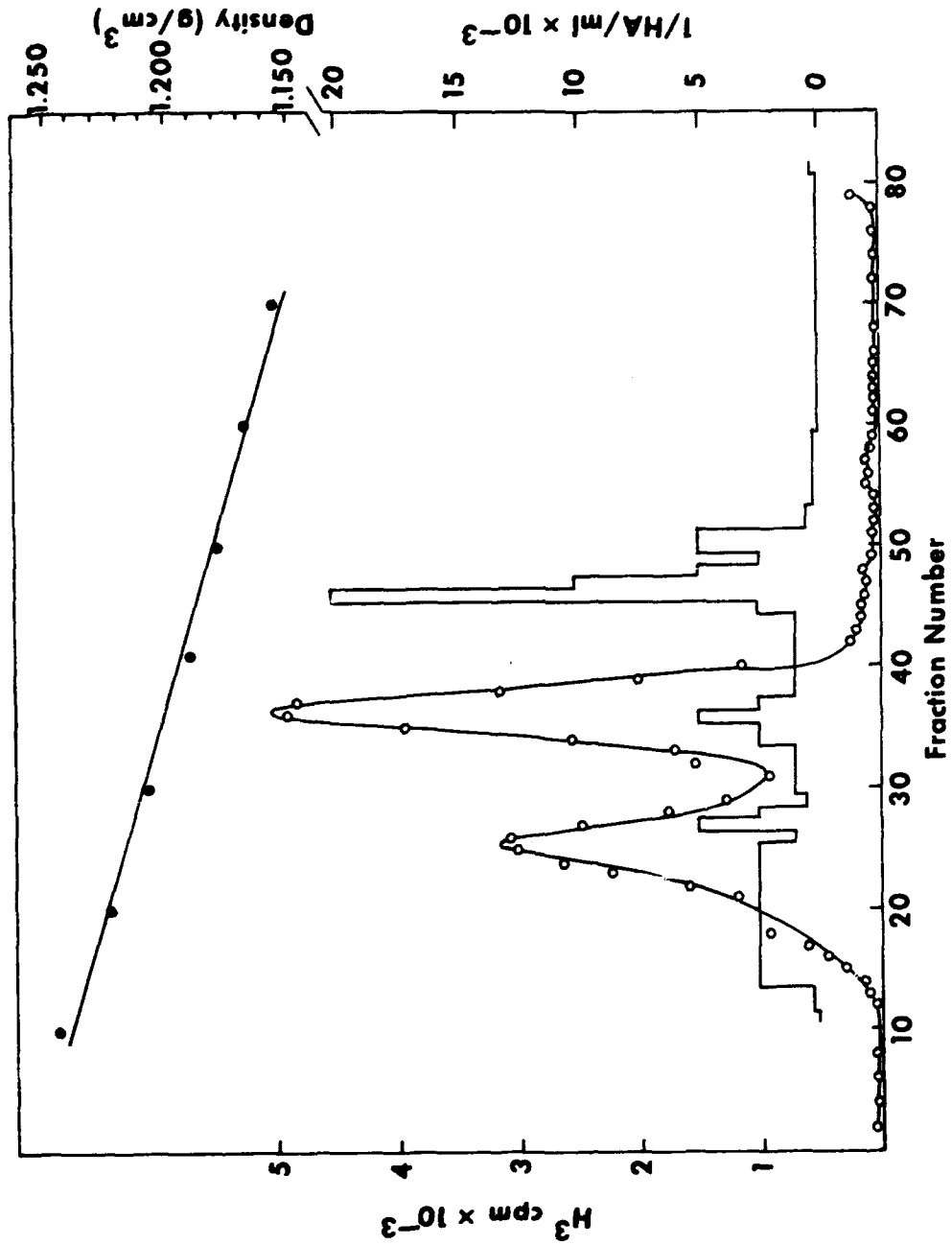

Figure 2. CsCl Density Gradient Centrifugation of Partially Purified EEE Virus Labeled with Uridine-H^3. Radioactivity (o), CsCl density (●), hemagglutinin titer (bar diagram).

The possibility of a contaminant was ruled out because fresh, plaque-purified EEE virus contained infectious virus of $\rho = 1.23$. Secondly, evidence was obtained against the presence of a density mutant in the population by performing a rebanding experiment. The virus was fractionated in a CsCl gradient, and virus that banded at each of the three densities was plaqued on chick embryo monolayers. Virus from the resulting plaques was used to infect additional monolayers, and the progeny virus was subjected to a second CsCl centrifugation. Results from this experiment indicated that EEE virus populations of heterogeneous densities could be obtained from plaques of EEE virus fractionated at significantly different densities.

The presence of an immature or subviral EEE virus particle in the two infectious bands was considered. Experiments presented elsewhere,[9] in which pfu titers in isotonic and hypertonic assay systems were compared, could not differentiate between EEE virus of $\rho = 1.20$ and $\rho = 1.23$. These experiments were based on the fact that the ratios of plaquing efficiencies under these two assay systems are known to differ markedly when comparing intact virus with some subviral forms.[10]

Another rebanding experiment was performed to study the possibility that the EEE virion was breaking down during centrifugation in CsCl. EEE virus labeled with $P^{32}O_4$ was fractionated on a CsCl density gradient, and the virus material banding at each of the three densities was obtained. A second CsCl centrifugation was then performed, each band in a separate tube. The radioactivity profile obtained is shown in Figure 3. These data show that a majority of the most dense material rebanded at $\rho = 1.23$, but a small amount was detected at $\rho = 1.18$. On the other hand, virus taken from the mid-band appeared to break down, yielding radioactivity at each of the three densities, similar to the way freshly purified EEE virus behaved on an initial CsCl fractionation. The HA-containing band rebanded quantitatively at $\rho = 1.18$. The radioactivity present where the HA activity banded can be accounted for by the phospholipids that this virus is known to contain. Thus, it appears that EEE virus of $\rho = 1.23$ was produced by the salt-induced alteration or degradation of the complete EEE virion. We felt that the HA-rich material banding at $\rho = 1.18$ also resulted from salt-induced breakdown.

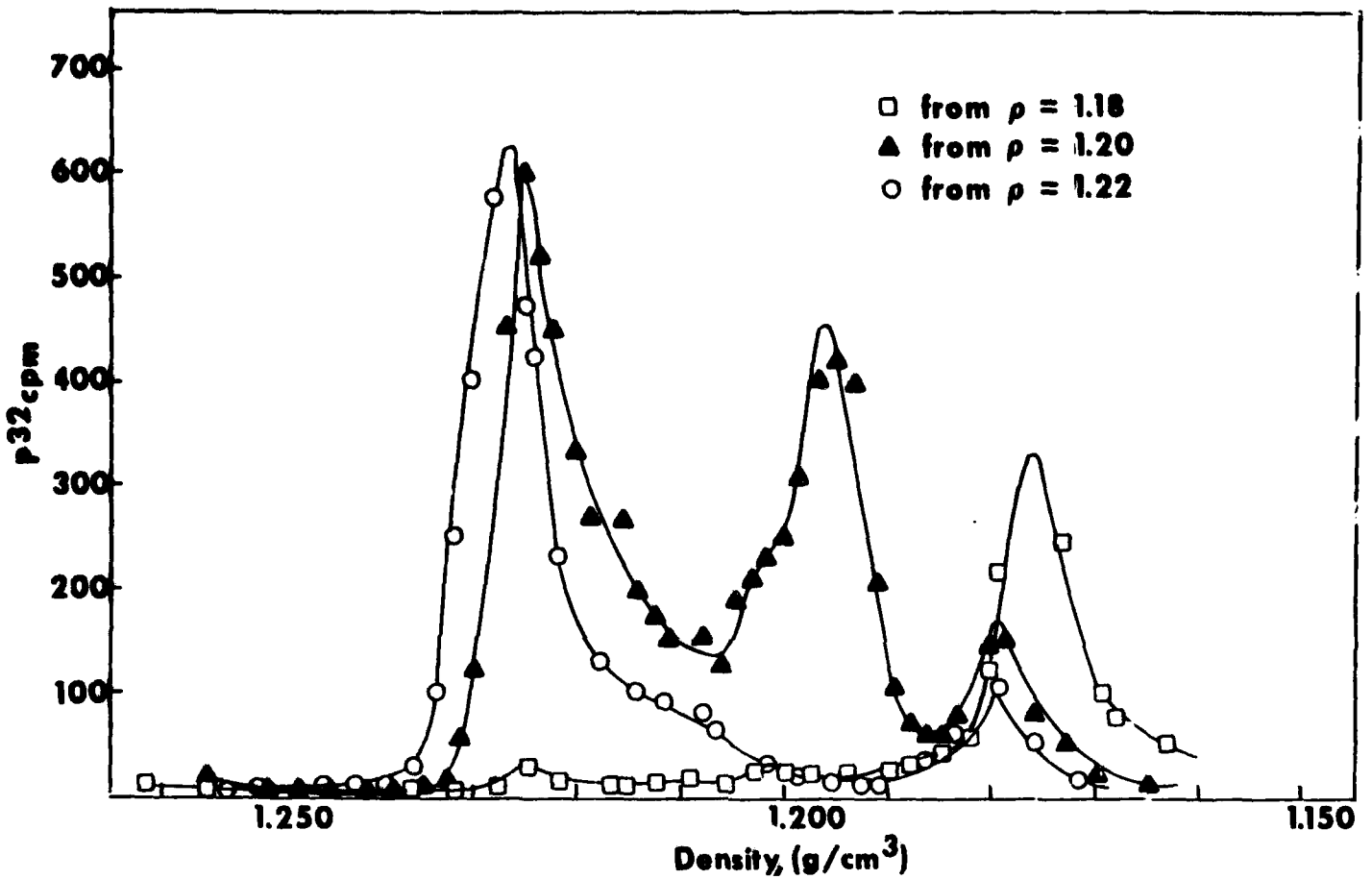

Figure 3. Rebanding of $P^{32}O_4$-Labeled EEE Virus Material of Densities ρ = 1.18, 1.20, and 1.22. Each band was fractionated from a CsCl gradient and rebanded in a separate tube: band ρ = 1.18 (□), band ρ = 1.20 (Δ), band ρ = 1.22 (o).

IV. DISCUSSION

The general structure of arboviruses, determined by negative-staining techniques, consists of a spherical core surrounded by an envelope, which may be composed, in part at least, of host-cell membrane material. In addition, short projections that appear more like a fringe than spikes may be discerned on the surface of the virion. Recent evidence[11] supports the possibility that the hemagglutinin of Semliki Forest virus may be carried on these projections because a loss of HA activity paralleled the removal of the projections. Virus treated so as to remove the projections was also shown to be infectious.

One explanation of the data in the present report is that the complete EEE virion is being degraded by the CsCl used to construct the density gradient. The HA activity detected at $\rho = 1.18$ may represent fragments of envelope together with projections stripped from the surface of the virion. This is consistent with a lighter buoyant density and the labeling pattern in Figures 2 and 3. Infectious virus banding at $\rho = 1.23$ could therefore be EEE virus that has lost some of its fringe. Such a loss might give rise to a denser particle if it became relatively less rich in lipid or its properties of hydration were altered. Particles in this density range still possessed the ability to hemagglutinate and are not therefore considered to be altogether stripped of fringe. Experiments are presently under way to examine each band by electron microscopy.

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14. Key Words
*Eastern equine encephalitis
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